The Genetic Structure of Population and Captive Breeding of the Black Clam, *Villorita cyprinoides* (Gray, 1825)

SYNOPSIS

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Chapter 1

General Introduction and Review of Literature

1.1 INTRODUCTION

The terms ‘ecosystem’ and ‘biodiversity’ are some of the most frequently used terminologies in biosciences. An ‘ecosystem’ is defined as a complex biological system composed of all the organisms found in a particular physical environment, interacting with it and with each other, and thus functioning together as a single unit (Bogers et al., 2019). On the other hand, ‘biodiversity’ or ‘biological diversity’ refers to the variability among organisms, including diversity within and between species and ecosystems (DeLong, 1996). Studies focused on biodiversity in the past, and information on ecosystem services (ES) was supplied through them (Valencia Torres et al., 2021).

The aquatic ecosystem, covering approximately 71% of the earth's surface includes seas, oceans, estuaries, and inland water bodies that host a vast majority of biodiversity (Takahashi et al., 2023). Among aquatic ecosystems, the marine ecosystem includes environments that comprise estuarine and coastal wetlands and offers indispensable ecosystem services (Figure 1). The estuarine environment is a semi-enclosed coastal water body that connects to the sea with variable salinity due to the influence of adjacent marine and freshwater bodies (Whitfield & Elliott, 2011). This dynamic ecotone connecting the sea and the freshwater body harbors rich and unique biodiversity. This in turn offers an exclusive array of diverse resources and essential services to mankind. However, the global decline in estuarine and coastal ecosystems (ECEs) is affecting several critical ecosystem services, thus becoming one of the most heavily exploited ecosystems worldwide (Thrush et al., 2014; Booi et al., 2022). Estuaries are widely acknowledged for their production of shellfish, which can be harvested by cultural, recreational, and commercial fishers as well as through aquaculture (Thrush et al., 2014). This practice not only supports the local economy and preserves cultural traditions, but also serves as a fundamental source of sustenance.

In 2022, worldwide exports of bivalves (clams, scallops, and mussels) reached a total of USD 6.0 billion, accounting for approximately 3% of the global export value of aquatic animal products (FAO, 2024), elucidating its astounding potential as a food resource of international importance. By weight, they also account for 20% of the entire production

of aquatic animals (FAO, 2018).

India possesses over 10% of the world's aquatic biodiversity, particularly in fish and shellfish species, establishing itself as a significant global contributor to marine life (Mahanayak & Panigrahi, 2021; NFDB, 2024). Hence, the total fish production of India in 2023 amounts to 17.545 million tonnes, with marine fish contributing 4.432 million tonnes and inland fish production reaching 13.113 million tonnes (GoI, 2023). The average yearly production of bivalves from 1996 to 2000 was approximated at 0.152 million tonnes, while the average currency exchange (Forex) from 1991 to 2003 on bivalve and gastropod exports amounted to 130 million Indian rupees (Kripa & Appukuttan, 2003; Mohamed, 2013). In addition, the shift from capture to culture-based fishing in inland fisheries has paved the way for a sustainable blue economy in the country (IBEF, 2022). These small-scale fisheries (SSFs) operate in coastal to estuarine waters, and employ women, youth, and the elderly, thereby supporting their livelihoods (Anderson et al., 2011).

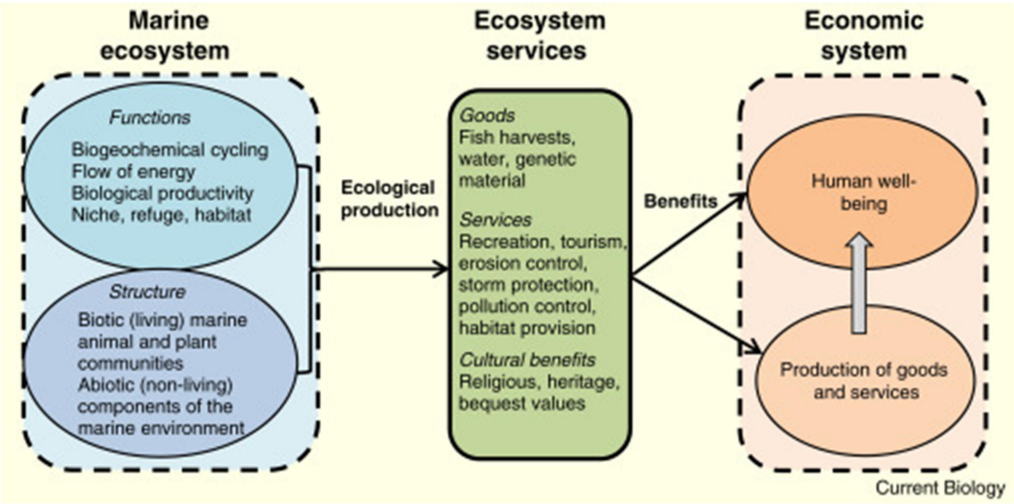

Figure 1: Overview of ecological services derived from the marine ecosystem (Barbier, 2017).

In India, the bivalve fishery resources are dominated by two clam species, the Indian black clam and the short-neck clam (CMFRI, 2018). The Indian black clam *Villorita cyprinoides* accounts for more than 66% of the clam production in India (CMFRI, 2021), whereas, 80% of the export is dominated by short-neck clam. The majority of the capture of the earliest is sourced from Vembanad Lake, Kerala, India, and the latter is sourced from Ashtamudi Lake, Kerala, India. There are many reports regarding the

overexploitation of black clam. However, various regulations have been undertaken on the fishery of short-neck clam in Ashtamudi Lake thus, the Ashtamudi clam fishery was certified with Marine Stewardship Council (MSC) fishery standards, certifying the fishery is sustainable and the product was labelled with blue MSC label (http://ashtamudi-stories.msc.org/). However, there arises a conflict in the scientific naming of Ashtamudi's short neck clam. On the other hand, no significant molecular works have been carried out with Indian black clam with the given exploitation rate.

1.2 OBJECTIVES OF THE STUDY

Research on the genetic and molecular characteristics of *Villorita cyprinoides* has been scarce, notwithstanding their overexploitation due to economic and ecological significance. However, considering the current overexploitation and alarming population decline it is highly essential to undertake molecular genetic studies and breeding standardization in black clam. Thus, this study aims to bridge the above gaps by furnishing comprehensive data using multidisciplinary fields and thereby providing insights into the taxonomic reassessment, population structure, mitochondrial genome, evolutionary history, early embryogenesis, and diet analysis of *V. cyprinoides*. In addition to the major objectives, a minor objective to clarify the taxonomic resolution of India's Ashtamudi short-neck clam was added, as proper species identification is fundamental to all scientific research. Hence to address the above research gaps, the specific objectives of the study are delineated as follows:

1. To identify whether different sub-populations of *Villorita cyprinoides* Gray, 1825 exist throughout the animal distribution range.
2. Mitogenome profiling of *V. cyprinoides*, comparative mitogenomics and phylogenomics of Imparidentia.
3. Characterization of complete mitogenome of India's Ashtamudi short neck clam, comparative mitogenomics, and phylogenetic analysis with special emphasis on sub-family Tapetinae.
4. *De novo* transcriptome assembly of *V. cyprinoides* and phylotranscriptomics of Heterodonta (Class: Bivalvia)
5. Induced spawning, larval rearing, and innate diet analysis of Indian black clam.

1.3 SPECIES OVERVIEW

1.3.1 Overview of *Villorita cyprinoides* (Indian black clam)

Villorita Gray, 1833, is a genus within the family Cyrenidae Gray, 1840. This genus is typically regarded as monotypic, with *Villorita cyprinoides* Gray, 1825, being the sole recognized species (Lydeard & Cummings, 2019). Cyrenidae is a diverse family with various species, some of which are economically important and invasive. The family is known for having brackish water genera and for species that have adapted to both freshwater and brackish water environments. (Graf, 2013). Within Cyrenidae, there are three genera: *Batissa* J. E. Gray, 1853; *Corbicula* Megerle von Mühlfeld, 1811; and *Villorita* J. E. Gray, 1833. (MolluscaBase, 2024).

1.3.2 Overview of *Marcia recens* (short-neck clam)

Superfamily Veneroidea Rafinesque, 1815 has long been a source of scientific contention due to its intricate phylogenetic inter-relationships (Keen, 1969). Within the Veneroidea, Tapetinae Gray, 1851 emerges as a subfamily characterized by rapid evolution, remarkable homogeneity in characters, and morphological plasticity resulting in species misidentification and yielding perplexing phylogenetic outcomes among its 14 subfamilies (Chen et al., 2011). Within Tapetinae, the genus *Marcia* H. Adams & A. Adams, 1857 was established, with *Venus opima* Gmelin, 1791 assigned as the 'type by subsequent designation' (MolluscaBase eds., 2024).

In India, Subba Rao records 17 species of Tapetinae from Indian waters, and two species were collected from the Ashtamudi Lake. *M. recens* and *M. opima*. (Rao, 2017).

1.4 REVIEW OF LITERATURE

1.4.1 Genetics in Fisheries

Capture fisheries and aquaculture play a crucial role in ensuring food security, improving nutrition, and maintaining the well-being of communities mainly in rural areas (Thompson & Subasinghe, 2011). Since the 1980s, concern and awareness regarding fisheries resources have increased due to a decline in total landings (Watson & Pauly, 2001). This resulted from increasing fisheries exploitation, habitat destruction and fragmentation, environmental pollution, and increased mortality rates due to climate change and related issues (Kenchington, 2003; Pauly et al., 2005). Thus, aquaculture

emerges as a potential solution for overfishing, reducing pressure on wild stocks and meeting expected protein demand (Longo et al., 2019). Aquaculture, however, confronts numerous challenges and difficulties, including the lack of genetically modified organisms (GMOs), the need for species-specific diets, and high disease-related death rates (Yue & Wang, 2017). These challenges have been addressed by applying genetic principles and methods to improve the fish breeding program and develop appropriate wild population management to archive and maintain genetic diversity (Frankham, 2010; Lind et al., 2012; Valenzuela-Quiñonez, 2016).

1.4.2 Research work on *Villorita cyprinoides*

Cyrenidae which constitute a diverse range of species with differing attributes (e.g. economically important along with invasive taxa) is known for the retention of brackish water genera and parallel radiation of species to freshwater and brackish water environments (Graf, 2013). *Villorita cyprinoides* (Gray,1825), the Indian black clam, a bivalve belonging to the family Cyrenidae inhabiting on or even below the surface of bottom sediment in the brackish water ecosystem, is endemic to the peninsular India (Ansari et al., 1981). Even though the brackish water biome is the natural habitat of *V. cyprinoides*, the wide salinity tolerance of this resilient species enables its range expansion to the freshwater environment (Arun, 2009), as exemplified in Vembanad Lake (Kerala, India) seasonally divided by the Thanneermukkom Barrage.

The genus *Villorita* embracing *V. cyprinoides* is traditionally considered to be monotypic (Lydeard & Cummings, 2019) and acclaimed as prime species in Indian clam fisheries, prominently cherished for both meat and shell contributing more than 2.3 million USD to the economy in 2018-2019 (Vijaya Krishna & Ammini, 2018). Despite the tedious collection and processing exercise for the marketing of black clam the species always maintains a supreme demand in the market due to its nutritive value (Joy & Chakraborty, 2017). The use of the species for local consumption, export, and protein supplement in shrimp and poultry feed, makes the natives depend mainly on the clam for their sustenance and livelihood (Vipinkumar et al., 2017). The calcium-rich shell, a by-product in clam fishery is a potential raw material for the manufacture of lime and cement. The recent study that revealed the presence of antioxidative and anti-inflammatory metabolites from *V. cyprinoides* (Joy & Chakraborty, 2018) opens up new avenues of research in nutraceuticals. Apart from the top-notch economic significance,

bioremediation, biocontrol of phytoplankton as well as biomonitoring of estuarine heavy metal pollution is feasible through *Villorita* (George et al., 2013).

The fragmentary decline in the population of *Villorita cyprinoides* in certain regions was found in past decades (CMFRI, 2018). The reproductive success of the black clam depends on salinity and the decline in the clam population on the southern side may be correlated with the drop in salinity associated with creating a freshwater environment during monsoon (Suja & Mohamed, 2010). The detrimental effect of the decline in black clam population is affecting the livelihood of people depending on clam fishery urged the Govt. of Kerala to take immediate actions for rehabilitation through clam relaying (G.O(Rt)No. 460/16/F&PD of Fisheries & Ports (C) Department, Thiruvananthapuram, dated 01.08.16) to replenish the stock to ensure a sustainable supply of food, as implemented in many developed countries (Muehlbauer et al., 2014). Also, the dredging for the sub-fossil deposit of clamshell for industrial use (Kripa et al., 2004), pollution due to effluents from shrimp processing plants, eco-tourism, urban and agricultural runoff and infestation of the invasive *Eichhornia crassipes* engender threats to the clam (Laxmilatha & Appukuttan, 2002) in its natural habitat.

The economic and ecological eminence of *Villorita cyprinoides* results in continuous demand leading to overexploitation of the species including juveniles. Extensive harvesting of clam from the wild, niche alterations, and exploitation of juveniles could be a potential threat to the existence of the species (Vidya et al., 2017). The selective pressure applied in stock by overfishing cannot be reversed through genetic selection on phenotypic traits (Law, 2000). In-depth studies on the exploitation of juvenile black clams suggested the implementation of Minimum Legal Size (MLS) in clam fishery to reduce economic loss and preserve genetic variance (Suja & Mohamed, 2014). The ecosystem degradation as well as overexploitation, if continued at the present level, may result in the reduction of effective population size leading to increased homozygosity, reduction in fitness due to faster erosion in genetic variation of related traits, and ultimate genetic drift in far or near future. Along with genetic information, the need for breeding standardisation and proper farming techniques is inevitable for a sustainable black clam fishery.

Villorita cyprinoides, a resource of paramount importance in meeting the country's need for a cheap and balanced diet, has received less attention at the genetic level. The

present status of the black clam fishery demands genetic investigation and analysis for the proper implication of management and conservation.

1.4.3 Research work on *Marcia recens*(=*Paphia malabarica*)

The taxonomic nomenclature of the Ashtamudi short-neck clam, also known as the yellow-foot clam, remains a subject of ongoing debate, prompting numerous studies to address the issue (Arathi et al., 2018, 2019; Sukumaran et al., 2019). Extensive research has been conducted on the growth and population dynamics of the short-neck clam, contributing significantly to our understanding of the species (Mohite & Mohite, 2009; Thomas & Nasser, 2009).

Investigations into the development of primary cultures of mantle cells (Dessai, 2012), and the effects of media additives on biomineralization have provided valuable insights into the physiological processes of the short-neck clam, with potential applications in aquaculture (Niyoyitungiye et al., 2021).

The nutritional quality and antioxidative metabolites of the short-neck clam have been thoroughly studied, highlighting its potential as a source of functional foods and its importance in human nutrition (Pawar et al., 2013; Joy & Chakraborty, 2017). Research on gonadal development, condition index, and percentage edibility has further enhanced our understanding of the specie's reproductive biology and its suitability for consumption (Mohite et al., 2008; Mohite & Mohite, 2009).

Environmental factors such as salinity and pH have been shown to significantly influence larval development and spat production (Gireesh & Gopinathan, 2004), while biochemical changes during larval development have been documented, providing insights into the species developmental biology (Gireesh et al., 2009). Further research on the impact of microalgal diets on larval growth and survival, contributing to the understanding of optimal conditions for larval rearing and aquaculture practices was also conducted (Gireesh & Gopinathan, 2008).

The fishery and biology of *Paphia malabarica* have been well-documented (Thomas et al., 2003; Kavitha et al., 2021). The fishery of *P. malabarica* from Ashtamudi estuary, Kollam, Kerala has been certified by MSC as sustainable and the fishery (Mohamed & Malayilethu, 2015).

Chapter 2

Population genetics and shell geometric morphometrics of the Indian black clam V*illorita cyprinoides* across the Indian coastline

2.1 INTRODUCTION

The exploitation rate of the Indian black clam, *Villorita cyprinoides*, is increasing due to its increase in demand. This may be due to the heavy exploitation of the species even in the juvenile stage (*mallikakka*) (Suja & Mohamed, 2014; Nagampoozhi, 2020). The mean size of the clam in the fishery has decreased from 28.4 mm in 2016 to 28.1 in 2017 (CMFRI, 2018). To minimize the extinction probabilities of a population (Saccheri et al., 1998) and ensure the survival of threatened species (Spielman et al., 2004) maintenance of genetic diversity is essential. Information on population genetic structure could be useful for fishery management measures, including breeding and conservation. Population genetic studies provide identification of good population from which suitable broodstock can be maintained for aquaculture practices. Lack of information on the genetic structure may lead to overexploitation and subsequent collapse of the resource. No studies on the genetic structure of the genus *Villorita* is recorded so far.

Apart from that, the genus *Villorita* has been the subject of interesting controversy for its systematic position and scientific name. Different species (*V. corbiculoides, V. cornucopia, and V. cyprinoides*) of the genus *Villorita* are described (Rao, 1989). Later, it was concluded that these species may be junior synonyms of *Villorita cyprinoides*. Even though *Villorita cyprinoides* is accepted as a monotypic species, two variants of the species have been recorded to date, *Villorita cyprinoides* var. *cochinensis* (Hanley) and *Villorita cyprinoides* var. *delicatula* (Preston). They differ from each other only in the general shape of the shell and the hinge teeth. Considering the economic and ecological importance of *Villorita*, there is an urgent need to fix the taxonomic uncertainty of the species.

2.2 REVIEW OF LITERATURE

Molecular genetic markers are useful tools for measuring genetic variation and gene flow among populations. There are three classes of genetic markers that are regularly used in population genetic analysis and phylogenetic studies; allozymes, mitochondrial

DNA, and nuclear DNA (Liu & Cordes, 2004; Schlötterer, 2004; Avise, 2012). The selection of appropriate markers that are to be used to determine genetic variation is a prerequisite for population genetic study.

The mitochondrial DNA (mt DNA) reveals high levels of sequence diversity at both intra and interspecies levels, despite great conservation of gene function and arrangement (Avise et al., 1983). Mt-DNA is extensively used for population genetic studies, owing to its properties of the maternal mode of inheritance, rapid divergence, high rate of linkage, and lack of recombination. Different regions of mitochondrial DNA *viz.* cytochrome c oxidase subunit I (COI, cox1), D-loop, and cyt b have been used for determining the population structure in bivalves (Jackson, 1986; Zhan et al., 2009; Ye et al., 2015). The mitochondrial gene is considered to be fast evolving than nuclear genes. The more rapidly evolving region would refine the picture of gene flow within species.

2.3 MATERIALS AND METHODS

Specimens of *V. cyprinoides* were collected from six different locations Muhamma and Vaikom [Vembanad Lake, Kerala]; Chettuva [Chettuva Estuary, Kerala]; Mangalore [Netravathi, Karnataka]; Goa, Madgao [Zuari estuary, Goa]; and Tamilnadu [Pulikat lake, Tamilnadu] along the Indian coast. Total DNA was extracted from the adductor tissue using the Qiagen DNeasy Blood and Tissue Kit (QIAGEN, Valencia, CA, USA). The universal primer set HCO2198: TAAACTTCAGGGTGACCAAAAAATCA, LCO1490: GGTCAACAAATCATAAAGATATTGG (Folmer et al., 1994) was employed to amplify the partial COI region. Parameters of genetic diversity, including the number of haplotypes, haplotype diversity (hd), nucleotide diversity (π), and the number of polymorphic sites (s) were generated using DnaSP 6.12.01 (Librado & Rozas, 2009). ARLEQUIN v3.0 (Excoffier et al., 2005) calculated the Pairwise F-statistics (F_{ST}) (Weir & Cockerham, 1984) to assess population differentiation. TCS haplotype network (Clement et al., 2000) diagram was constructed using POPART software (Leigh et al., 2015).

For geometric morphometric analyses, each right valve was photographed on the inner side. On each image, 11 landmark points (Zelditch et al., 2012) were located on the position of internal features of the shells, and 19 sliding semilandmarks on the shell

outline were marked with tpsDig2 to quantify shape (Rohlf, 2005). The shell morphometric study with 240 specimens collected along the Indian peninsula was performed using linear discriminant analysis.

2.4 RESULTS

In linear discriminant analysis, the first two canonical discriminant functions account for 77% of total variability with LD1 contributing 48% and LD2 with 31%. It is revealed that there is a distinct separation of clam population between some locations (Figure 2). However molecular analysis employing the COI barcodes observed a low genetic divergence, that does not exceed the intraspecific threshold limit established for molluscs. This result overrules the existence of new species in the genus *Villorita* other than *V. cyprinoides,* thus creating a morpho-molecular discordance between the morphometric and molecular species boundaries. The change in morphology is not consistent or does not result from genetic variation between populations rather, this discordance may be due to the plasticity exhibited by the species in adapting to environmental changes. Hence the distinct clam population could be different morphological forms/morphotypes of the same species, *V. cyprinoides*.

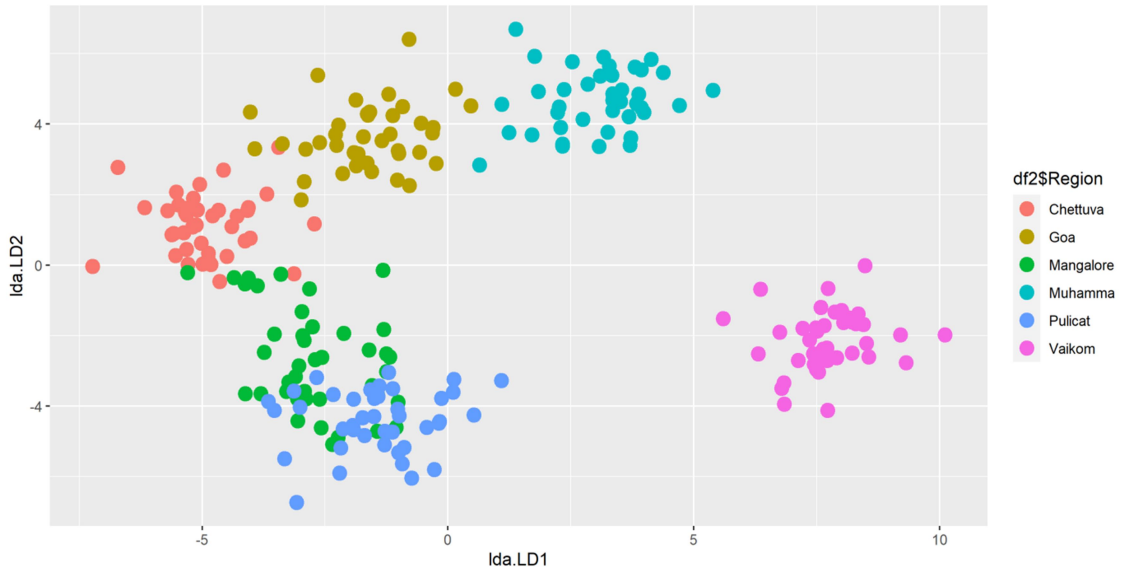

Figure 2. Canonical discriminant function score plot of *Villorita cyprinoides*, based on shell morphometric data collected from six locations, visualized using the ggplot2 package in R software.

A phylogenetic analysis based on Bayesian inference was performed to confirm the

results derived from GMA. The phylogenetic tree distinctly separates the black clam from other species within the family Cyrenidae, which served as the outgroup. The sequences from *V. cyprinoides* divide into two major clades, where the second major clade further diverges into two, forming three lineages. Each clade exhibited strong posterior probability values nearing 1, indicating high confidence in the separation of these evolutionary lineages.

The genetic distance between Lineage 1 and Lineage 2 is 1.5%, while the distance between Lineage 2 and Lineage 3 is slightly lower at 1.4%. The smallest genetic distance, 1.3%, was observed between Lineage 1 and Lineage 3. All the genetic divergence values indicated a close genetic inter-relationship between the lineages.

After analyzing 187 sequences from six populations, 84 haplotypes were identified with Haplotype diversity (Hd)= 0.96. AMOVA analysis revealed the global F_{ST} value as 0.28 suggesting the high genetic diversity within the population. The highest pairwise F_{ST} value noticed was 0.63, between Mangalore and Vaikom populations. On the other hand, the lowest value observed was 0.026 between Goa and Vaikom (Table 1).

Location	Chettuva	Muhamma	Vaikom	Goa	Mangalore	Tamilnadu
Chettuva	0.00					
Muhamma	0.099*	0.00				
Vaikom	0.089*	0.099*	0.00			
Goa	0.036	0.035	0.026	0.00		
Mangalore	0.54*	0.45*	0.63*	0.53*	0.000	
Tamilnadu	0.16*	0.034	0.16*	0.09*	0.48*	0.00

Table 1. Pairwise F_{ST} values after the global analysis of molecular variance (AMOVA) analysis after Bonferroni correction. * represent $p < 0.05$.

The TCS haplotype network diagram revealed three distinct haplogroups (Figure 3). Among the three haplogroups, one major group comprised individuals from all locations, forming a distinct population. Of the two minor haplogroups, one group included individuals from all locations but with fewer haplotypes, while the other haplogroup was predominantly made up of haplotypes from Mangalore. The star-shaped pattern of the major haplogroup was observed with a central/ancestral haplotype and the peripheral haplotypes have minimal variation with the central haplotype. Hap 7 is the

central haplotype with the highest frequency and widest distribution, whereas the peripheral haplotypes have with lowest frequency and restricted distribution.

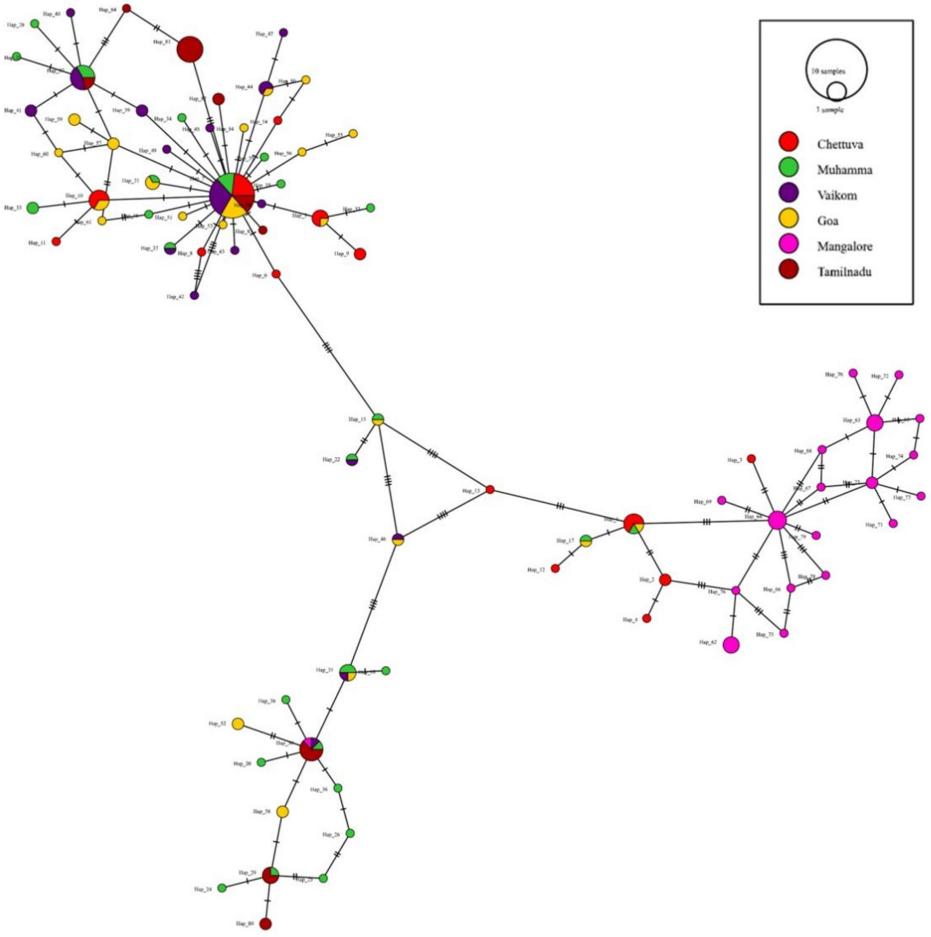

Figure 3: The haplotype network diagram generated using TCS method in POPART.

2.5 DISCUSSION

The alpha taxonomy of the genus *Villorita*, like many bivalve species, has customarily relied on traditional morphology for species identification. However, the combination of morphometric analysis with molecular markers has considerably increased to aid species discrimination (de Souza & Caetano, 2020). Hence, in the present study, an integrative approach using GMA and molecular analysis was used to reassess the taxonomy of *Villorita*.

The GMA of clam shell shape effectively differentiated clams from each location, resulting in the formation of six separate groups. However, phylogenetic analysis using

the COI gene divided the phylogram into three lineages, highlighting the genetically distinct lineages within *V. cyprinoides*, which were not fully captured by the morphometric data. The results from GMA and molecular studies (both phylogeny and genetic divergence) created a morpho-molecular discordance.

Based on the genetic diversity, haplotype network diagram and AMOVA results the existence of genetic structure within the black clam population was detected. The TCS haplotype network diagram for the black clam population can't assort haplotypes according to sampling areas, indicating a genetic similarity among these estuarine populations

2.6 CONCLUSION

The results showed the importance of integrative taxonomic approaches in resolving species boundaries in the genus *Villorita* and underscored the population genetic structure of Indian black clam *Villorita cyprinoides*. Given that the phenotypic plasticity, a prominent feature of mollusc adaptation to environmental conditions, relying solely on morpho-species approaches to delineate species can lead to an overestimation of true biodiversity and population. The population genetic diversity addressed in this chapter successfully identifies genetic variation within different populations which is critical for sustainable resource management.

Chapter 3

Mitogenome profiling of *V. cyprinoides,* comparative mitogenomics and phylogenomics of Imparidentia

3.1 INTRODUCTION

Although there have been notable advancements in the field of phylogenetics in recent decades, the fundamental evolutionary relationships within Bivalvia remain unresolved. Because of great diversity and the lack of systematic studies, bivalves have a confusing taxonomic history, and thus their interrelationships remain poorly understood (Chen et al., 2011). Inconsistent results of phylogeny and systematics are presented in different malacological literature (Giribet & Wheeler, 2002).

The families Cyrenidae (Order: Venerida) and Sphaeriidae (Order: Sphaeriida) are heterodont bivalves that have independently adapted to freshwater environments within

Imparidentia (Park & Foighil, 2000). Cyrenidae constitutes over 100 species with diverse range of species with differing attributes from economically important along with invasive taxa (Graf, 2013).

Villorita cyprinoides, a resource of paramount importance in meeting the country's need for a cheap and balanced diet, has received less attention at the genetic level. The present status of the black clam fishery demands genetic investigation and analysis for the proper implication of management and conservation. Hence it is necessary to determine the mitogenome architecture of *V. cyprinoides* thereby delivering ample molecular data for genus *Villorita.*

3.2 REVIEW OF LITERATURE

Mitochondrial DNA (mt-DNA), the lone double-stranded circular extranuclear genome in eukaryotes prevailed by endosymbiosis, is of crucial importance because of its ATP synthesis in the cell through oxidative phosphorylation (Martijn et al., 2018). Besides energy production, small size, high copy number, conserved genes, high rate of evolution, low recombination, and maternal inheritance enable mitochondria as a potential marker in molecular studies (Mitchell, 1961; Fernández-Pérez et al., 2017a). Bivalve mitogenome as metazoans typically consists of 13 protein-coding genes for OXPHOS, 2 rRNA, 22 tRNAs, and an A+T rich region controlling replication and transcription (Boore, 1999). However, peculiar mitochondrial features like extensive genome reorganisation and occurrence of Doubly Uniparental Inheritance (DUI) in certain bivalves extended supplementary information on evolutionary patterns across species and among conspecifics (Boore & Brown, 1998; Guerra et al., 2017; Froufe et al., 2020). The popularity of mitogenome as a marker of choice for phylogenetic studies is on arise due to a surge in published mitogenomic data which provides improved phylogenetic resolution (Lee et al., 2019). Nevertheless, molecular studies employing combined mitochondrial and nuclear markers played a pivotal role in imparting accurate and refined knowledge on bivalve molecular systematics, phylogenetics, population health analysis, and comparative phylogenomics (Sharma et al., 2013; Fernández-Pérez et al., 2017b).

3.3 MATERIALS AND METHODS

Mt-DNA was isolated and paired-end sequencing library was prepared using QlAseq

FX DNA Library Kit. Adapter-ligated libraries were PCR amplified, quantification and size evaluation of the library was performed in Bioanalyzer 2100 using High Sensitivity DNA chip (Agilent Technologies) and sequenced on Illumina sequencing platform (Illumina HiSeq 2500, USA). Raw sequences generated after sequencing (2x150 PE) were retrieved in the FASTQ format. High-quality reads were subjected to d*e novo* assembly using CLC workbench 6 (Qiagen, Germany) with a read map back algorithm. The scaffold identified as mitogenome of *Villorita cyprinoides* was annotated using MITOS2 (Bernt et al., 2013). Protein-Coding Genes (PCGs) were identified using the online ORF Finder Tool (https://www.ncbi.nlm.nih.gov/orffinder/).

Ribosomal and transfer RNAs were confirmed using NCBI BLAST (http://www.nchi.nlm.nih.gov/BLAST/) tRNAScan-SE v2.0 (Lowe & Eddy, 1997) and ARWEN v1.2 (Laslett & Canbäck, 2008) searches. DNAPlotter (Carver et al., 2009) was used to generate a circular visual overview of the annotated mitogenome. Conserved gene order analyses of mt-genomes at the superfamily level were conducted in superorder Imparidentia. Nucleotide composition statistics of PCGs excluding stop codon and mitochondrial ribosomal RNAs were calculated separately by Geneious v11.1.5 (Kearse et al., 2012). Codon usage bias and Relative Synonymous Codon Usage (RSCU) of protein-coding genes were inferred using the codon usage table computed through DnaSP v5.10.01 (Librado & Rozas, 2009). AT and GC skew were analysed by the formula AT skew = (A+T)(/A-T) and GC Skew = (G+C)/(G-C) (Perna & Kocher, 1995). The phylogenetic position of *V. cyprinoides* concerning the superorder Imparidentia was appraised. Appropriate subset partitions and evolutionary models for genes were selected by PartitionFinder v.1.1.1 (Lanfear et al., 2012). Phylogenetic analysis using the Maximum likelihood method was inferred using IQ-tree with 1000 ultrafast bootstrapping replicates (Nguyen et al., 2014). Bayesian analysis was implemented using MrBayes v.3.2 (Ronquist et al., 2012). The estimation of divergence time between species and clade was determined using Bayesian relaxed clock models and MCMC algorithms (Yang & Rannala, 2006; Rannala & Yang, 2007) implemented in the mcmc tree programme of PAML software v4.9 (Yang, 2007).

3.4 RESULTS

The two mt-genomes of *Villorita cyprinoides* lineages are 15,880bp and 16030bp in length, comparable to previously reported bivalve mitogenomes. Gene content of

V. cyprinoides displayed 37 typical sets of genes found in metazoan mitogenome (Boore, 1999) constituting 13 protein-coding genes (PCGs); encoding three electron transport chain (ETC) complexes (nd1-nd6, nd4l, cytb& cox1-cox3) and F_0F_1 ATP synthase complex (ATP6 & ATP8) in OXPHOS. Besides PCGs, two ribosomal RNA (rRNA) and 22 transfer RNA (tRNA) genes were observed with the putative control region (CR). All the 37 genes are located on the major (H-strand) strand with forward orientation like in other Heterodont bivalves (Yuan et al., 2012). Figure 4 plots the circular map of the black clam mitogenome.

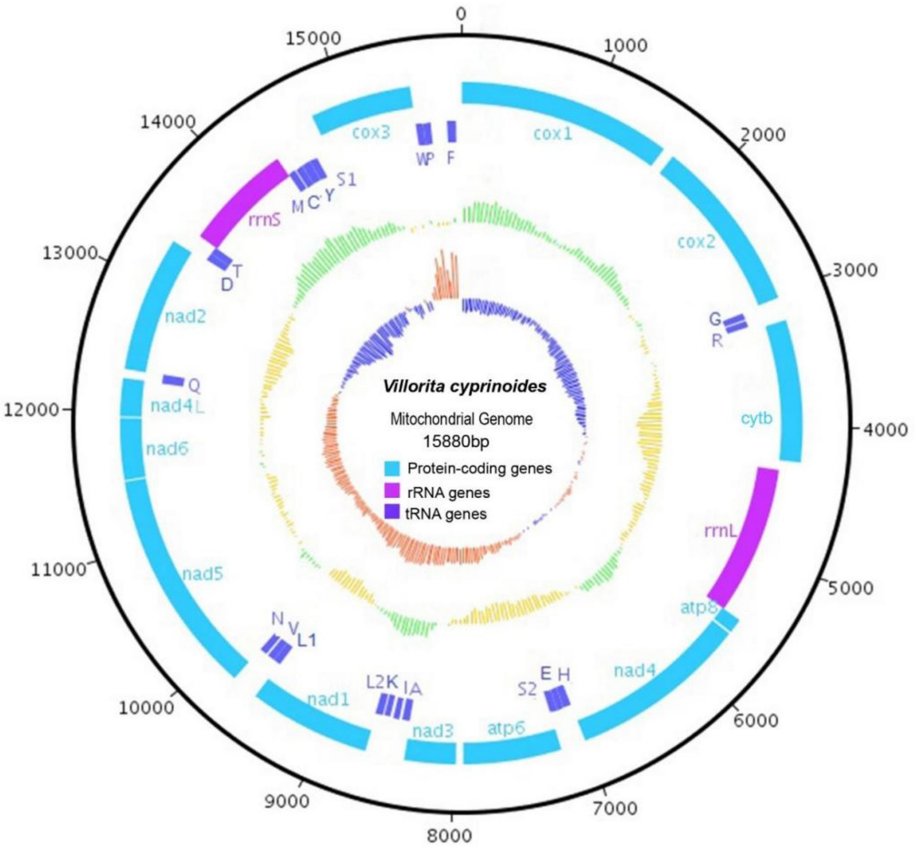

Figure 4. Mitochondrial genome organisation of *Villorita cyprinoides* visualised with DNA Plotter.

Gene rearrangement, accepted as a marker of common ancestry, (Boore et al., 1998) varies unpredictably owing to single strand gene distribution in bivalves (He et al., 2011). The gene rearrangement explained through the TDRL (Tandem Duplication Random Loss) model is believed to transpire in bivalves as in vertebrates (Yu et al., 2008). Though overall gene order is not well conserved within bivalve superfamilies,

gene arrangement is substantially similar after excluding tRNA genes. Conversely, gene arrangements in Imparidentian bivalve families are nearly identical except for tRNA duplication, and translocation of genes, but variable between families. The conserved gene order or synteny blocks displayed in superfamilies are shown in Figure 5.

Phylogenetic estimation incorporating the subset partitions and evolutionary models for both Maximum likelihood (ML) and Bayesian Inference (BI) analysis generated a well-resolved tree with similar topology. All major clades were well supported with posterior probability >95 and bootstrap >85. The combined tree with node label showing posterior probability (PP) values and bootstrap (BS) replicates is displayed in Figure 6. The phylogeny of Venerida, the most debated order in the monophyletic subclass Heterodonta, is still undergoing extensive revision (Plazzi & Passamonti, 2010; Bieler et al., 2014; González et al., 2015). The present study considered 47 species, including 30 Venerida, 16 Cardiida, and one Myida confirmed the monophyly of the Venerid family, Cyrenidae (Park & Foighil, 2000). Family Cyrenidae (Superfamily: Cyrenoidea) form a well-supported clade corroborating monophyly, placing *G. coaxans* towards the stem, and *V. cyprinoides* and *C. fluminea* to its crown. Cyrenidae co-clusters with Arcticoidae establishing Arcticoidae as an immediate sister taxon, whereas Vesicomyidae (Glossoidea) lineage forms a separate clade.

The multi-gene phylogram was employed for molecular dating, Figure 7. The early Jurrasic fossil record of *Corbicula* sp. (201-170 mya) was used to establish the primary appearance of Cyrenid clam (Poulton, 1991). According to the dated tree, diversification of Cyrenid clams from genus *Geloina* occurred roughly around the early Jurassic, 179 mya (HPD, Highest Posterior Density: 169-198 mya) whereas, the evolutionary split between *Corbicula* and *Villorita* lineage appeared during early Cretaceous, 124 mya (HPD: 56-173 mya).

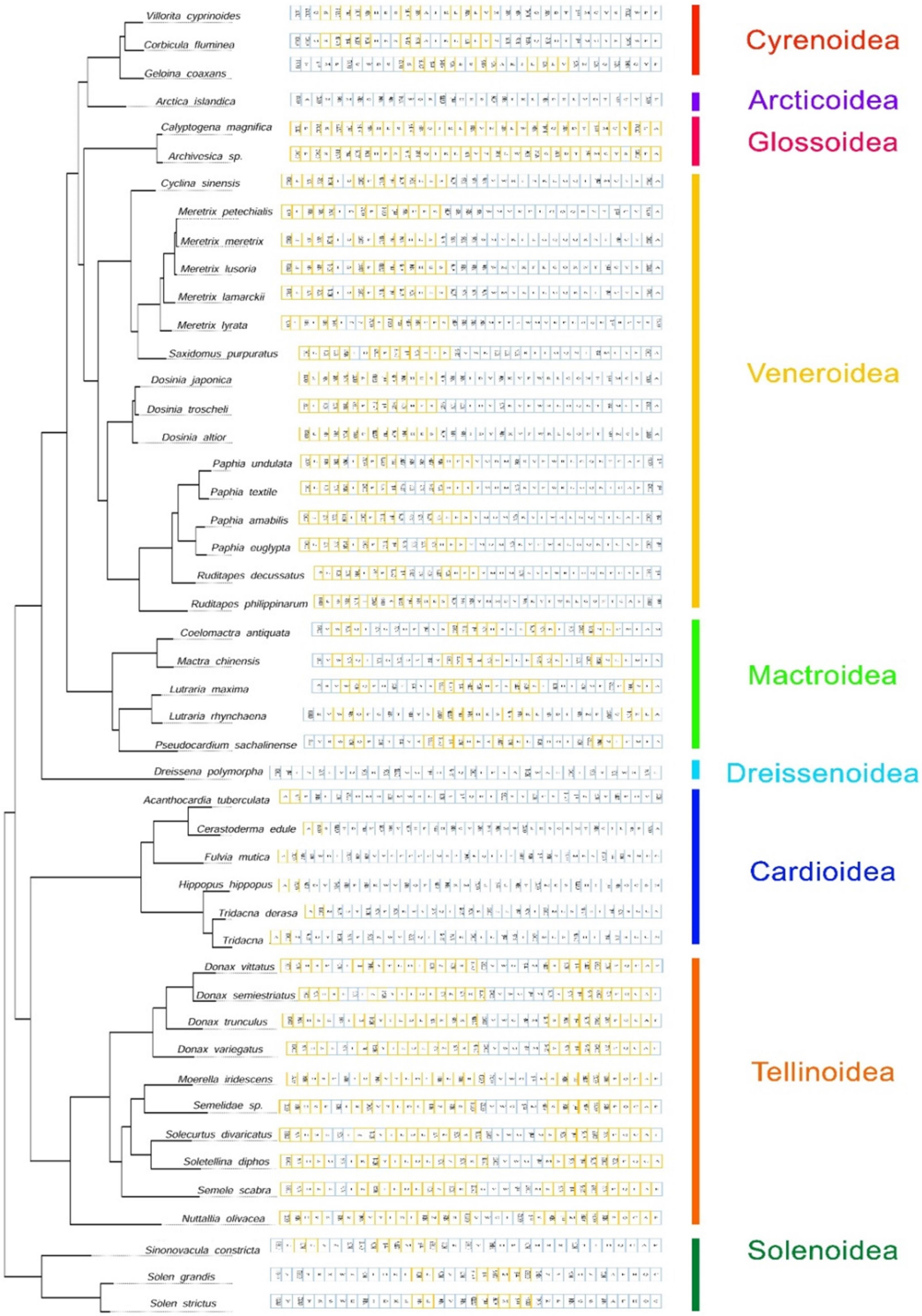

Figure 5. Linear illustration of Imparidentian mitogenomes superimposed on phylogenetic tree comparing synteny blocks in superfamily level. Tangerine colour bar depicts conserved gene order or synteny blocks

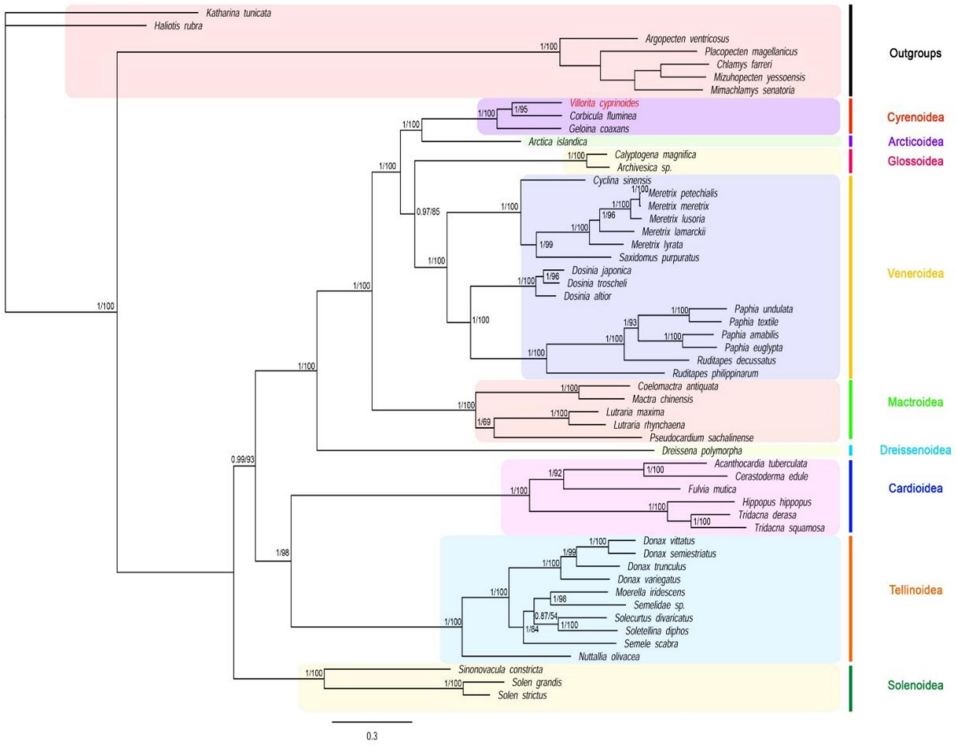

Figure 6. Phylogram evaluated based on nucleotide sequences of multilocus data from 47 species of superorder Imparidentia.

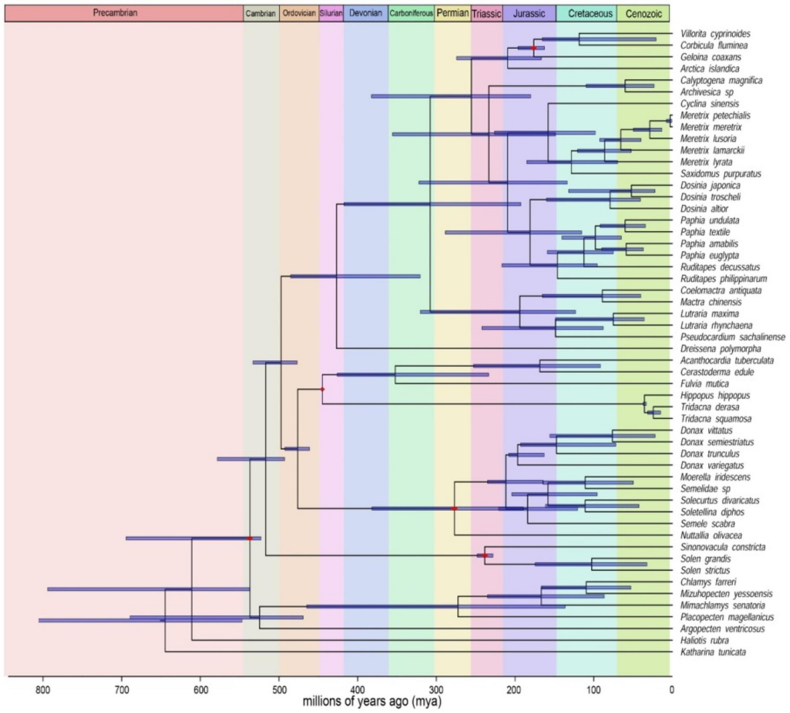

Figure 7. The time-calibrated multi-gene tree topology along with the geological time scale (mya). Red circles denote the fossil calibration points

3.5 DISCUSSION

The length of the *V. cyprinoides* mitogenome was comparable to previously reported bivalve mitogenomes with gene content displaying 37 typical sets of genes found in metazoan mitogenome (Boore, 1999) and H-stand located genes like in other Heterodont bivalves (Yuan et al., 2012). Gene rearrangement, accepted as a marker of common ancestry, can vary unpredictably owing to single-strand gene distribution in bivalves (Boore et al., 1998; He et al., 2011). This gene rearrangement can be explained through the TDRL (Tandem Duplication Random Loss) model, which is believed to transpire in bivalves as in vertebrates (Yu et al., 2008).

The confirmation of Cyrenidae's monophyly strengthens its classification as a distinct evolutionary lineage within Venerida. The placement of *Geloina coaxans* at the base of the Cyrenidae family tree suggests it is an earlier diverging member, while the positioning of *Villorita cyprinoides* and *Corbicula fluminea* at the crown indicates a more recent common ancestor for these species. The timing of the evolutionary split between the *Corbicula* and *Villorita* lineages during the Early Cretaceous, aligns with known evolutionary patterns within the genus (Wang et al., 2023).

3.6 CONCLUSION

Even though enormous mitogenomes of clams have been characterised, there is a dearth of molecular data for economically and ecologically significant clam, *Villorita cyprinoides*. The present study determined the first complete mitochondrial genome of a multivalent endemic black clam, *V. cyprinoides*, which can be considered a keystone species that can perform top-down and bottom-up control as indicated by Gallardi in 2014. Mitogenome sequences can be utilised for comparative phylogenomic studies within family Cyrenidae and development of genetic markers for identifying cryptic species if any. Apart from this, the generated mitogenome will act as a baseline data for population genomics studies. The validation of monophyletic status of Cyrenidae enriches the transparency of phylogenetic status, yet integrated investigation of other Cyrenid clams with dense species sampling is required to conserve our conclusion and to provide evolutionary divergence among and between taxon.

Chapter 4

Mitogenomic profiling and phylogenomics of India's Ashtamudi short-neck clam

4.1 INTRODUCTION

Precise species identification is an initial step in scientific research, particularly in fields of ecology, conservation biology, population studies, aquaculture, and proper implementation of fisheries management plans (Slade et al. 2017; Kürzel et al. 2022). Misidentification, on the other hand, can lead to a range of issues, including erroneous population monitoring, ineffective management plans, and unintentional harm to endangered species (Roberts et al., 2010). The Ashtamudi short neck clam (SNC) also known as the yellow foot clam, *Paphia malabarica* is an important Tapetinid bivalve contributing to significant clam fishery in India that holds a certificate from Marine Stewardship Council (MSC) for sustainability (http://ashtamudi-stories.msc.org/). This clam has been the subject of intense scrutiny and scientific debate surrounding its proper scientific name.

Veneroidea Rafinesque, 1815 (class Bivalvia) has long been a source of scientific contention due to its intricate phylogenetic inter-relationships (Keen, 1969). Within the Veneroidea, Tapetinae Gray, 1851 emerges as a subfamily characterized by rapid evolution, remarkable homogeneity in characters, and morphological plasticity resulting in species misidentification and yielding perplexing phylogenetic outcomes among its 14 subfamilies (Chen et al., 2011). Previous studies on short neck clam, re-evaluated its taxonomic classification, and synonymity of *Paphia malabarica* to *Protapes gallus* (Gmelin, 1791) (Sukumaran et al., 2019). Recently an approach using conchological character re-assigned *Paphia malabarica* (Chemnitz, 1782) (=*Protapes gallus* (Gmelin, 1791) as *Marcia recens* (Holten, 1802) which was later on validated using single gene marker (Arathi et al., 2018, 2019).

4.2 REVIEW OF LITERATURE

The advancement of molecular techniques has revolutionized the field of taxonomy, allowing for the integration of molecular evidence alongside traditional taxonomic characters (Johnson & Gosliner, 2012). Among the various molecular markers available, the mitochondrial genome has gained prominence in systematic and phylogenetic analyses of species due to its unique features, including a high copy

number, non-recombinant nature, fast evolutionary rate, gene order, and gene homology (Galtier et al., 2009; Ghiselli et al., 2021). Due to these characteristics, mitogenomes have been established as effective tools for taxonomic reconstruction and are utilized in the recovery of internal nodes at the family level in Mollusca (Plazzi et al., 2016; Ghiselli & Milani, 2019; Liu et al., 2022). Moreover, recent technological advancements, such as next-generation sequencing, have made it more accessible and affordable to sequence the entire mitochondrial genome (Sullivan et al., 2017; Wang et al., 2019). Consequently, the expanding availability of mitogenomes has significantly enriched nucleotide databases for bivalve species.

4.3 MATERIALS AND METHODS

DNA library preparation of an individual from Tuticorin (typotype) was performed using the TruSeq Nano DNA Sample Prep Kit (Illumina), and library validation was conducted using High Sensitivity D1000 Screen tape on the Agilent TapeStation. Low coverage whole genome sequencing (LC-WGS) was carried out using the Illumina NextSeq platform (Illumina, USA) with 2X150 bp paired-end (PE) chemistry, resulting in approximately 16 GB of data (~3X coverage).

The low-quality reads, with less than minimum Phred score of 20 were removed using Trimmomatic v0.39 (Bolger et al., 2014). Mitogenome assembly was performed using Geneious v. 11.1.5 (Kearse et al. 2012). Gene prediction in the assembled mitogenome was conducted using DOGMA (Wyman et al., 2004) and MITOS (Bernt et al., 2013). The curated genes were further analyzed using ExPASy (Artimo et al., 2012) and MEGA X. To identify transfer RNA (tRNA) genes, tRNAscan-SE 2.0 (Lowe & Chan, 2016) and ARWEN (Laslett & Canbäck, 2008) were employed. The presence of ribosomal RNA (rRNA) was confirmed using RNAmmer 1.2 (Lagesen et al., 2007). Additionally, the non-coding control region (NCR) was analyzed using Tandem Repeats Finder (Benson, 1999). The circular mitogenome was visualized using the CGView Server (Grant & Stothard, 2008).

The phylogenetic inferences were based on concatenated nucleotide of the 12 mitochondrial protein-coding genes (PCGs) and 2 ribosomal RNAs (rRNA). Complete mitogenome sequences of 47 venerida species were collected from the GenBank database (https://www.ncbi.nlm.nih.gov/genbank/). The best-fitting models of sequence

evolution were determined using PartitionFinder 2.1.1 (Lanfear et al., 2017). The software tool IQ TREE v1. 6.12 was used for phylogenetic analysis using the Maximum likelihood (ML) method (Nguyen et al. 2014). Bayesian Inference (BI) phylogenetic tree searches were performed using MrBayes 3.2.7 (Ronquist et al., 2012) with four independent runs of 100 million generations, sampling every 1000 generations.

4.4 RESULTS AND DISCUSSION

The shell morphology of a representative specimen of *M. recens* from the type locality is shown in Figure 8. These shell characteristics resemble the description of *M. recens* specimens by Arathi et al. (2018) and Kongasa (2019). Furthermore, the presence of a horizontal pallial sinus, which is a morphological synapomorphic character of the genus *Marcia*, is absent in both *Tapes* and *Paphia*. Therefore, it is not possible to assign the short-necked clam to the genus *Paphia* or *Tapes*. Hence short neck clam herein after referred to as *M. recens*.

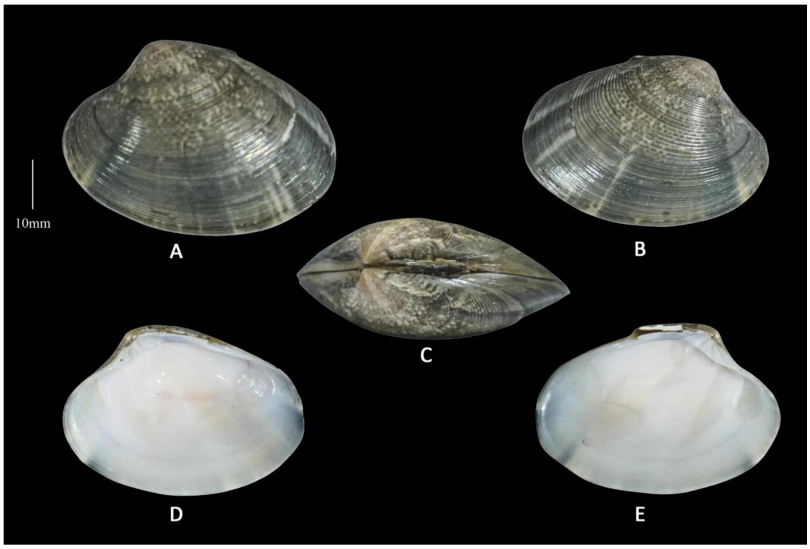

Figure 8. The shell characteristics of a representative adult specimen of *M. recens* (Holten, 1802) from the type locality. Major features of the shell show dorsal right valve (A), dorsal left valve (B), dorsal margin (C), internal view of right valve (D), and internal view of left valve (E)

The assembled mitochondrial genome of *Marcia recens* submitted under GenBank with accession number OR198073 was found to be 17991 base-pair (bp) in length. The generated mt-genome contained 13 protein-coding genes (PCGs), 22 transfer RNA (tRNA) genes, two ribosomal RNA (rRNA) genes (12S rRNA and 16S rRNA), and a

non-coding region (NCR) (Figure 9). A sequence homology search of the characterized mt-genome against the nucleotide databases showed a sequence similarity of 90.47% with the previous submission of *Marcia recens* (NC_068247.1) from China. The comparative mitochondrial gene order analysis within Tapetinae, revealed almost identical gene order within the genus level.

The concatenation of 12 protein-coding genes (PCGs) and 2 rRNA genes resulted in a dataset with a total length of 10,307 base pairs (bp). In genus *Marcia*, the species were nested within the genus *Tapes* and *Ruditapes* signaling the polyphyletic nature of the genus (Figure 10). *Marcia hiantina* and *Marcia japonica* form a sister relationship with *Tapes* with high posterior probability value, indicating its closer relationship with *Tapes*, whereas all the individuals from *Marcia recens* club form a single clade. *Marcia recens* form a major clade with *Ruditapes decussatus* showing *M. recens* was more closely related to *Ruditapes* than *Tapes* and *Paphia*. These phylogenetic classification in *Marcia* agrees with the result of gene order analysis.

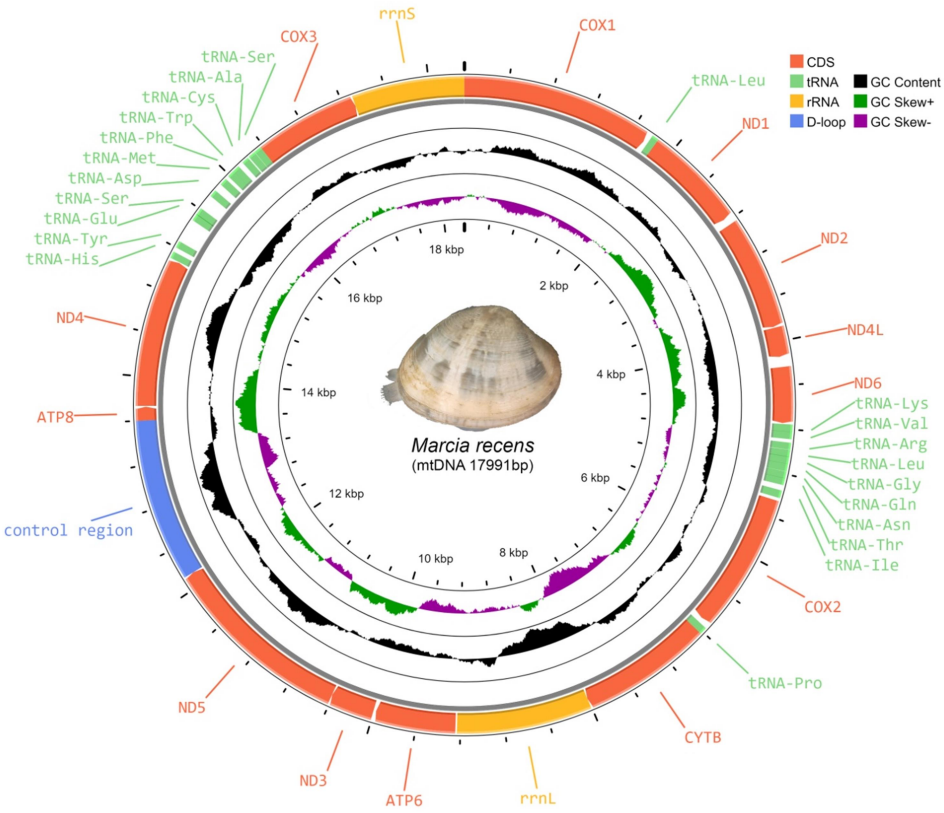

Figure 9. Circular mitochondrial genome map of the *Marcia recens* drawn by CGView

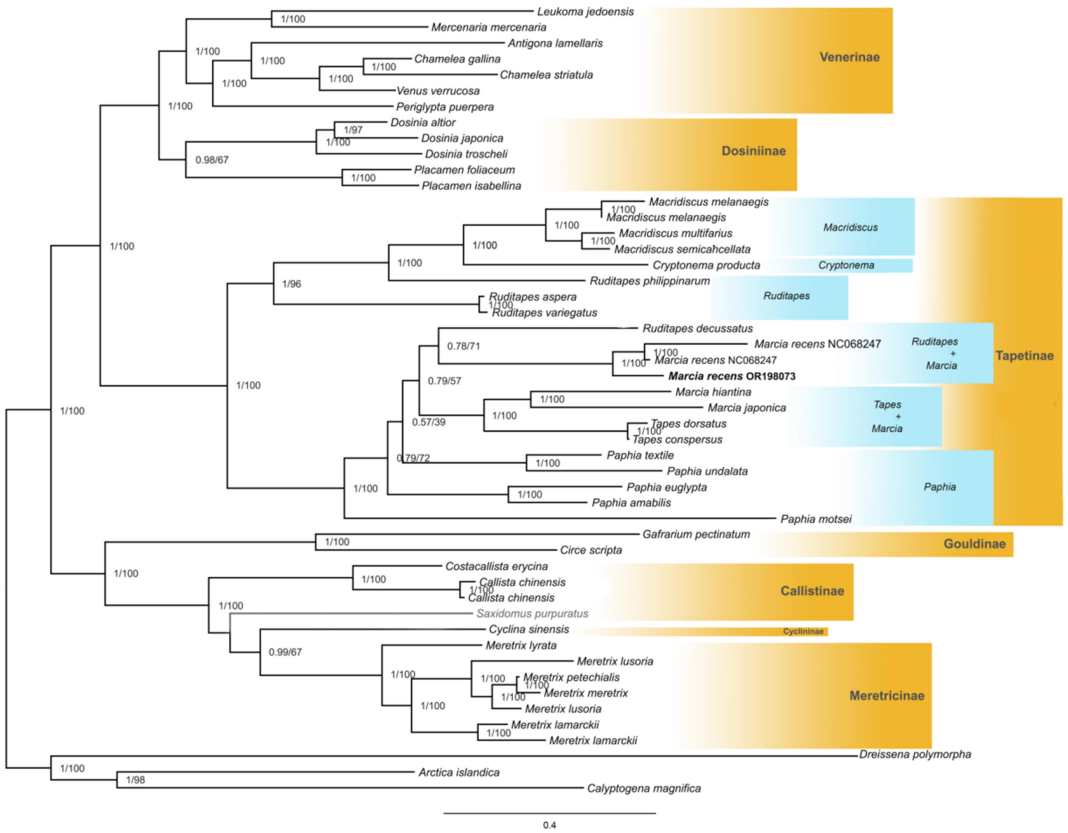

Figure 10. Phylogenetic tree generated using the Bayesian Inference based on the analysis of a concatenated dataset of 12 PCGs and two rRNAs.

4.5 CONCLUSION

The present study effectively resolves and validates the long-standing taxonomic uncertainties surrounding the Ashtamudi short neck clam (*Marcia recens*), offering valuable information critical for the proper management and conservation of the fishery resource. This also cuts down the chance of erroneous population monitoring and ineffective management plans Additionally, the present study fills the substantial gap in knowledge that has remained untouched for decades by generating mitochondrial genomic data for the species *M. recens* from Indian waters. Subsequent information generated through molecular and morphological analyses not only confirms the reassignment of the short neck clam as *Marcia recens*, but also offers further evidence to support the potential reassignment of *M. hiantina* and *M. japonica* to the genus *Tapes*. Also, the availability of the complete mitogenome opens up possibilities for comparative genome studies within the genus *Marcia*, enabling a better understanding of the evolutionary processes shaping this group of organisms. Overall, our findings

contribute to the field of molecular species identification and enhance our knowledge of the evolutionary dynamics within the genus *Marcia*. However, considering the polyphyletic nature of *Marcia*, we suggest that a dense molecular sampling within Tapetinae, along with all available species in *Marcia*, is still required to validate the phylogenetic status of the genus.

Chapter 5

De novo transcriptome assembly of *V. cyprinoides* and phylotrancriptomics of Heterodonta (Class: Bivalvia)

5.1 INTRODUCTION

Villorita is also considered an excellent sentinel organism, monitoring ecotoxicological changes and ensuring ecosystem health (George et al., 2013; Paul et al., 2015). Recent studies have discovered elevated levels of metals in black clams, leading to concerns about the overall health of the ecosystem (Neethu et al., 2021). Moreover, numerous earlier investigations have emphasized the substantial buildup and susceptibility of black clams to biologically essential metals like zinc (Zn) and copper (Cu) (Don Xavier et al., 2021; Raveenderan & Sujatha, 2011). Though they are reported to exhibit resilience to environmental perturbations, various climatic stressors are directly influencing their survival (Paul et al., 2021). To address these issues, molecular responses, fluctuations in the internal environment, signaling pathways and associated genes need to be monitored when clams are probed for pollutants and environmental stressors (Wang et al., 2009). This requires the elucidation of robust Reference Transcriptomic Datasets (RTD) that provide a basic notion of transcripts produced by a non-model organism (Morillon & Gautheret, 2019). Despite the absence of reference genome, the transcriptomic data obtained in this study will serve as an important genomic resource and act as a prerequisite for further ecotoxicological and gene expression studies. Conversely, the most recent phylotranscriptomics approach to reconstruct the phylogenetic relationship of Cyrenoidea within Euheterodonta. The phylotranscriptomic approach has recently shown potential for resolving deep nodes in bivalves (González et al., 2015; Moles et al., 2021).

5.2 REVIEW OF LITERATURE

Estuarine regions are ecotone zones with rich biodiversity that possess great ecological

and economic values (Barbier et al., 2011). Among the wider range of species communities, the malacofauna in the estuarine system represents a prominent fisheries resource, rendering a significant contribution to Small Scale Fisheries (SSF) (Ramachandra et al., 2012; Wijsman et al., 2018; Chuku et al., 2021). Being a biodiversity-rich ecosystem, the detrimental effects of climatic changes and anthropogenic pressures exerted on estuarine communities, particularly in bivalves, were implausible (Castro-Olivares et al., 2022). This inland fishery has been overexploited and reported rapid and fragmentary population decline despite implementing a minimum legal catch size of 10 mm as a preventive measure (Suja & Mohamed, 2010; Paul et al., 2021). Niche fragmentation, climate change and associated environmental stressors have drastically altered larval development and threatened the existence of this organism (Nagarathinam et al., 2021). However, due to limited genomic resources, it is difficult to ensure conservation and sustainability of *Villorita* given the current significant risk in its population. In addition, eventhough the phylogenetic backbone of bivalves are relatively resolved, there exist uncertainities within large clades. Orthology search in huge data sets like genomic and transcriptomic data is a reliable tool in phylogenetic analysis (Lemer et al., 2019)

5.3 MATERIALS AND METHODS

Total RNA from each tissue sample (gill, foot, adductor muscle, mantle, and gonad tissues from each clam) was extracted, purified, and quantified separately. 1.5 µg of RNA from the five tissues of each clam were pooled in equimolar concentration to prepare two RNA-seq libraries (VcypMt2, VcypVt2) using TruSeq Standard mRNA-seq library preparation kit following manufacturer's protocol (Illumina, USA). Barcoded libraries were then sequenced on Illumina NovaSeq 6000 platform with 150 base pair (bp) PE mode. D*e novo* transcriptome assemblies of clean reads were performed using Trinity v2.20 and rnaSPAdes v3.9 with a *k-mer* of 25 (Bushmanova et al., 2019; Grabherr et al., 2011). Software Bowtie v2.3.5 and Benchmark Universal Single copy orthologs (BUSCO), were employed to re-assess the quality and completeness of the assembled transcriptomes (Langmead & Salzberg, 2012; Simão et al., 2015). Open Reading Frames (ORFs) and coding regions from non-redundant transcripts were predicted by TransDecorder v3.0.0 with default parameters (Haas et al., 2013). Subsequently, predicted protein-coding regions were homology searched by

BLASTtx and BLASTp against NCBInr (https://www.ncbi.nlm.nih.gov/), UniProtKB (https://www.ebi.ac.uk/uniprot/), RefSeq (https://www.ncbi.nlm.nih.gov/refseq/) and Pfam databases with an e-value threshold of 1e-5 (Altschul et al., 1997; Finn et al., 2010). Annotations of Gene Ontology (GO) were also performed with Eggnog, KEGG, and Metascape (https://metascape.org/), which assigns transcripts to cellular components, cellular functions, and biological processes (Huerta-Cepas et al., 2016). Genes involved in zinc metabolism were identified by screening KEGG ontology (KO) mapped transcripts. Functional protein domains were then assigned using InterProScan v4.0 (Quevillon et al., 2005). GMATA (Genome-wide Microsatellite Analyzing Towards Application) was used to identify simple sequence repeats (SSR). Reference protein sequences of 39 molluscan species were downloaded from MolluscDB and NCBI databases. Transcriptome of 39 molluscan species were downloaded from MolluscDB and NCBI databases. These sequences along with *Villorita* protein set were used to identify Ortholog genes with OrthoFinder v2.5.4 (Emms & Kelly, 2019). Phylogenetic reconstructions were performed using aligned single-copy ortholog genes from all the species with the maximum likelihood (ML) method in IQ-TREE. The data analysis and visualization were done through R software using the packages viz., *ggplot2, tidyr, dplyr, stringi, plotrix, forcats, ggVennDiagram, venn, ggpolypath* and *RColorBrewer* (Rdc, 2010).

5.4 RESULTS AND DISCUSSION

Reference-free *de novo* transcriptome assemblies for the reconstruction of transcriptomes from high quality reads retained after filtration of raw reads from the two libraries. The initial *de novo* transcriptome assemblies with TRINITY generated an average of 142491 contigs with 1214 bp length whereas rnaSPAdes generated an average of 112588 contigs with 1133 bp length. Comparison on statistics of *de novo* transcriptome assemblies using Trinity and rnaSPAdes assemblers were shown in (Table 2).

A total of 47607 transcripts were identified as Coding Domain Sequences (CDSs) using TransDecoder. Of these, 37487 unique CDSs returned positive BLAST hits against any of the six databases; UniProtKB, NR, Pfam, KOG, KEGG, and RefSeq databases while, 9714 transcripts were annotated simultaneously by all the databases (Figure 11). Statistics of functional annotation towards different databases are summarized in

Table 3.

Assembly Statistics	**TRINITY**		**rnaSPAdes**	
	VcypVt2	VcypMT2	VcypVt2	VcypMT2
Assembly size (bp)	177139464	183586011	127649071	133110033
Total number of contigs (bp)	142491	154847	112588	120855
N50 (bp)	2588	2461	2618	2517
Mean contig length (bp)	1243	1185	1133	1101
Longest contig (bp)	27080	31762	29464	35015
GC content (%)	38.14	37.96	37.88	37.76
Filtering Statistics	VcypVt2	VcypMt2	VcypVt2	VcypMt2
Assembly size (bp)	176775746	124858206	117467820	122551596
Total number of contigs (bp)	142370	127309	104403	112260
N50 (bp)	2585	2588	2574	2470
Mean contig length (bp)	1241	1243	1125	1090
Longest contig (bp)	27080	31762	29464	35015
GC content (%)	38.14	38.14	37.90	37.76

Table 2. Statistics of *de novo* assemblies and quality filtering of *Villorita cyprinoides* transcriptomes were displayed.

Heavy and trace metal contamination due to anthropogenic activities impact the estuarine ecosystem adversely (Niu et al., 2021). The heavy metal concentrations in estuarine sediment and in bivalves are strongly correlated with zinc and copper as major contaminants (Luo et al., 2014; El Nemr et al., 2016). Repeated toxicology studies have consistently revealed elevated levels of zinc in black clams, raising significant concerns about the overall health of aquatic ecosystems (Don Xavier et al., 2021; Raveenderan & Sujatha, 2011). Protein homology search with KEGG database identified the presence of two family of zinc transporters, ZnT (Zinc transporter)/SLC30A and ZIP (Zrt/Irt like protein)/SLC39A, which plays a significant role in maintaining zinc homeostasis at the cellular level (Kambe et al., 2015; Kong et al., 2020). A total of 14063 SSRs were recognized from transcriptome data using GMATA.

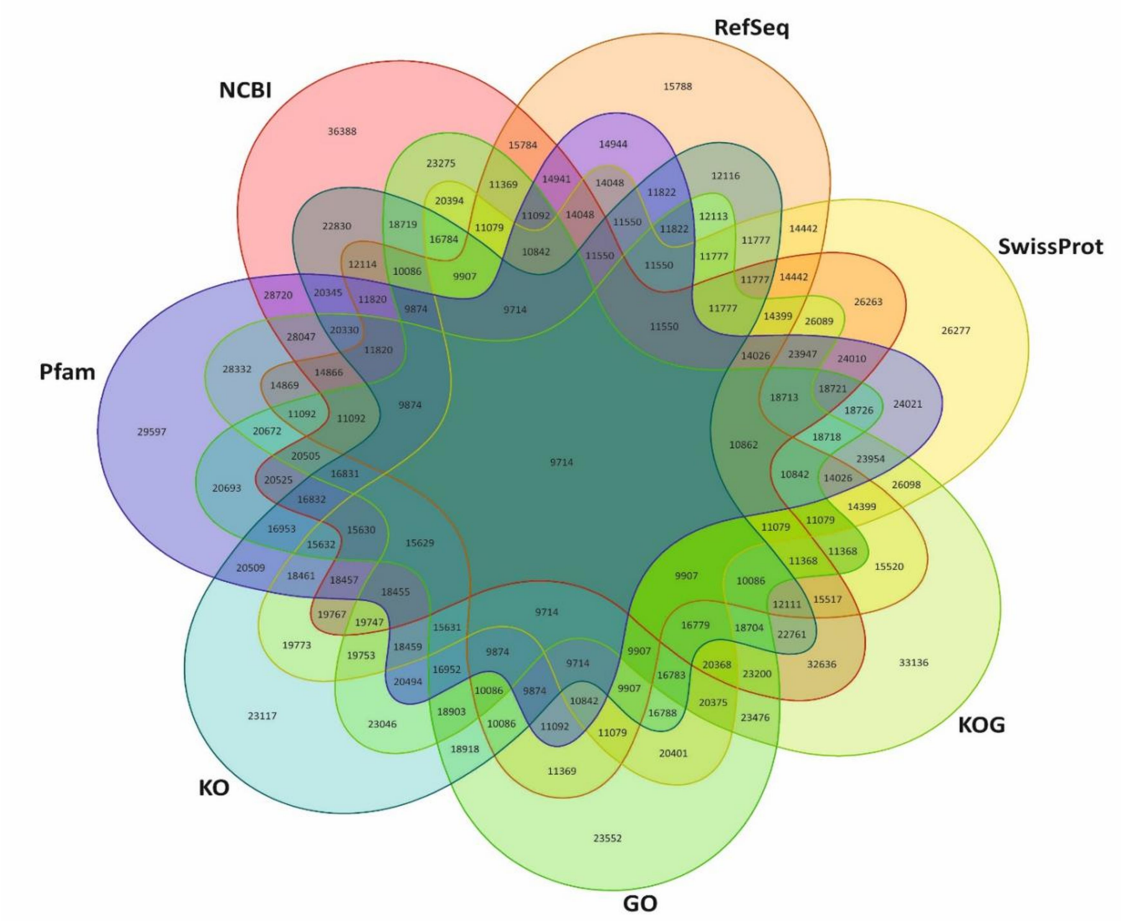

Figure 11. Venn diagram representing the functional annotation of genes in *Villorita cyprinoides* using six multiple public databases; NCBI, RefSeq, SwissProt, KOG, GO, KO, and Pfam.

Databases	Number of genes annotated	Percentage
NR	18795	39%
UniPortKB	23026	48%
RefSeq	15788	33%
Pfam	29597	62%
GO	23552	49%
KOG	33136	69%
KEGG	9098	19%

Table 3. Statistics of functional annotation by different databases.

The phylotranscriptomic approach has recently shown potential for resolving deep nodes in bivalves. A total of 40802 orthologous aminoacid regions identified from the assembled transcriptomes using OthoFinder were used for the phylogenetic reconstruction. The resulting topology using Maximum likelihood method (Figure 12)

corroborate with previous studies using transcriptomic data (Lemer et al., 2019). The analysis recovered the monophyly of superorder Anamalodesmata and Imparidentia with strong BS support (99.8/100).

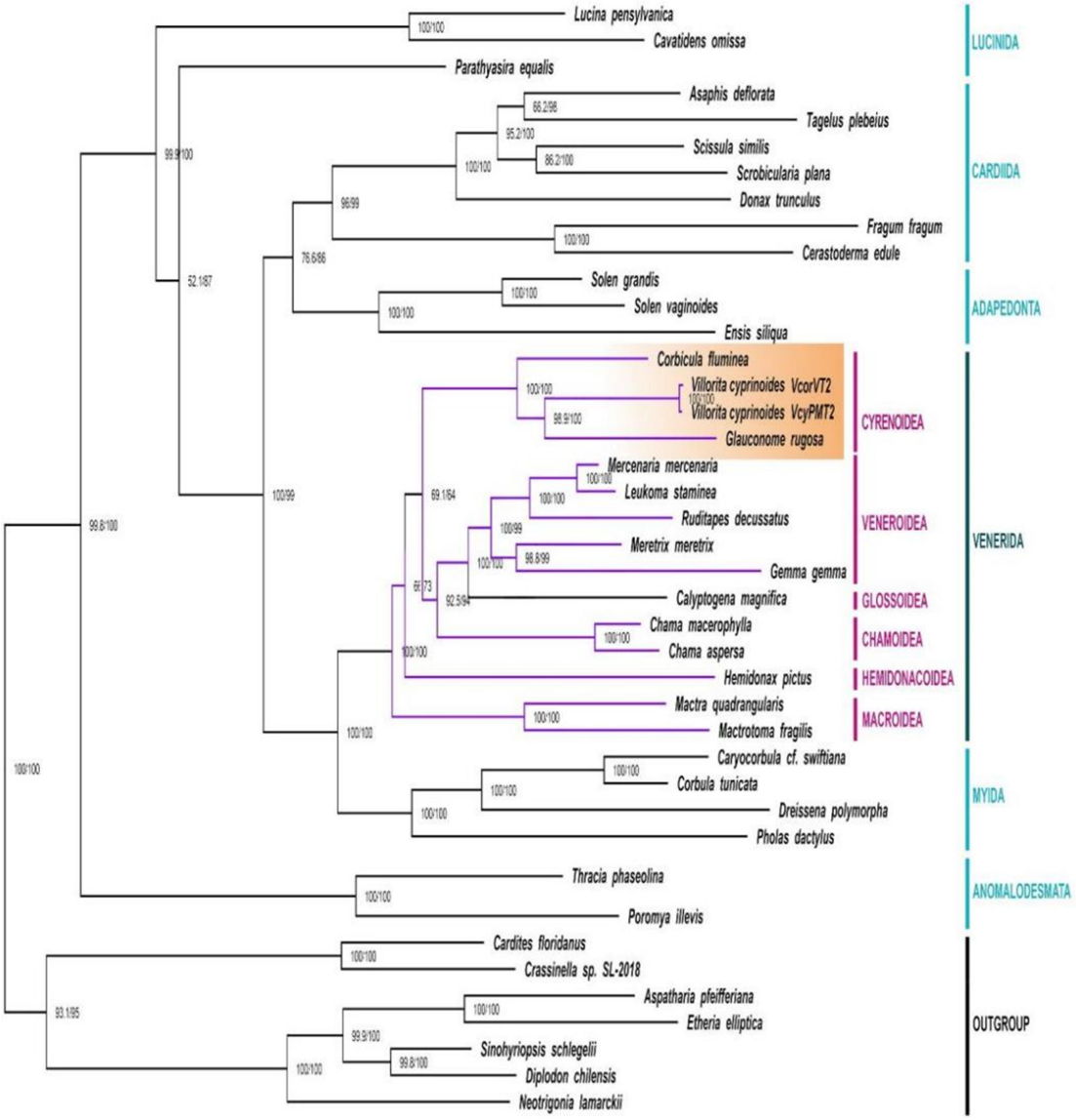

Figure 12. Phylogeny of Imparidentia based on 40802 aminoacid using IQ-TREE under Maximum Likelihood (ML) method.

5.5 CONCLUSION

The present study successfully established a transcriptomic reference profile for the sentinel species *V. cyprinoides*, thereby understanding the transcriptomic landscape of this species. Beyond the identification of key genes responsible for zinc metabolism and phylogenetic reconstruction through orthologous gene sets, the generated transcriptome

dataset holds great potential for future experimental studies aimed at elucidating the underlying processes of strategic adaptation of these bivalves to challenging environments. This study is not without limitations. Although the present study provides some potential biomarkers for ecotoxicological studies, further validation of these markers is essential for implementation in subsequent experimental studies related to specific physiochemical changes. Furthermore, as the transcriptomes of all Cyrenid species become accessible, the scope of comparative transcriptome studies can also be expanded to shed light on the evolutionary significance of this pivotal family. In addition, SSRs identified from the transcriptome profile of *V. cyprinoides* may serve as promising nuclear markers for future population genetic studies.

Chapter 6

Induced spawning, larval rearing, and innate diet analysis of the Indian black clam

6.1 INTRODUCTION

The conservation and sustainable management of aquatic biodiversity have become inevitable due to the increasing environmental and anthropogenic challenges (Dudgeon et al., 2006; Reid et al., 2019; Boyd et al., 2020; Hoffmann, 2022). Among the many species in the aquatic environment, the Indian black clam, *Villorita cyprinoides* Gray, 1825, is a native bivalve species found in the estuaries of the Indian Peninsula (Jones, 1969; Chatterji et al., 1984). The species has paramount significance as it serves as a valuable resource in meeting the country's demand for an affordable and nutritionally balanced diet. It thrives in sandy substrates further from the estuarine mouth, particularly in areas with low salinity (Suja & Mohamed, 2010). The black clam fishery contributes 45,000 tonnes which accounts for about two-thirds of the total clam production in India (66,000 tonnes) (CMFRI, 2018). In addition to its prominent economic value, *Villorita* plays a pivotal role as a sentinel organism, diligently monitoring ecotoxicological shifts and contributing significantly to the preservation of ecosystem health through its role as an ecosystem engineer (Theresa Paul et al., 2015). Recent decades have witnessed an increasing demand for commercialized species coupled with uncontrolled exploitation of wild stocks that has resulted in the collapse of natural resources, leading to the classification of some species as threatened. Standardizing the breeding of a new candidate bivalve species like *V. cyprinoides* requires a thorough understanding of its reproductive biology, diet, and embryonic and larval development.

6.2 REVIEW OF LITERATURE

In advanced shellfish aquaculture, multiple methods for induced spawning in molluscs have been developed to investigate reproduction, increase production, controlling the reproductive frequency for successful breeding cycles and production of selective strains (Achari, 1988; Sreenivasan and Rao, 1991; Aji, 2011; Bolasina, 2022). Spawning can be induced either by physical/conventional or chemical stimuli, with serotonin being a potent chemical trigger for promoting spawning (Gibbons and Castagna, 1984; Mies and Sumida, 2012; Nowland et al., 2021; Canesi et al., 2022; Funayama et al., 2022). However, studies integrating both physical and chemical stimuli to initiate spawning have not been investigated to date in black clams. Additionally, there is a dearth of research pertaining to the identification of the indigenous dietary preferences of the black clam.

6.3 MATERIALS AND METHODS

Mature male and female black clams was collected and the broodstock were conditioned for two weeks. A combination of physical and chemical methods was used to standardize induced spawning in black clam. Physical shock includes salinity and temperature shock whereas different concentration of serotonin (5–hydroxytryptamine creatinine sulphate complex, Sigma–Aldrich) was used for chemical induction. An optimal factorial design (4×3×2) in 33 runs was set up for the experimentation with the temperature ranges (A) from 22°C to 34°C with each block differing in 4°C (with levels: 22–26, 26–30 and 30–34), salinity ranges (B) from 0ppt to 20ppt with each block differing in 5ppt (with levels: 0–5, 0–10, 0–15 and 0–20) and the concentration of serotonin (C) ranging from 1.5–2.5mM (with levels: 1.5, 2 and 2.5) using Design Expert Software (Ver. 13) (Licensed to ICAR-CMFRI, Kochi) (Table 4).

Temperature range (°C) (A)	Salinity range (ppt) (B)	Concentration of Serotonin (mM) (C)
22-26	0-5	1.5
26-30	0-10	2
30-34	0-15	2.5
	0-20	

Table 4. Ranges of temperature (°C), salinity (ppt) and serotonin concentration (mM) tested to induce spawning of *Villorita cyprinoides* in hatchery

Fifteen numbers of conditioned clams were released into the initial experimental set-up. Once the animal began siphoning, the temperature and salinity were slowly increased as in the experimental design. 0.4ml of serotonin was injected intramuscularly two hours after the physical parameters reached its maximum to stimulate spawning. Water samples were collected from the bottom of the tank every 10 minutes after spawning to document early embryonic development. The response (Fertilized egg) generated from 33 trials were subjected to Analysis of Variance (ANOVA) and the significant effects/interactions were noted. The data were transformed using logarithmic transformation of the form $log(X+1)$ to make it amenable to ANOVA.

Water samples of 500ml were collected from the broodstock collection sites at three different depths of the surface, middle and bottom layers and thence combined into one integral sample which is then filtered in 0.22µm pore size membrane filter (Millipore, USA). The 18S (Product size ~350bp) region of 18S RNA gene was amplified using specific primers. The purified amplicons were subjected to index PCR using Nextera XT indices kit (Illumina, USA). Libraries were quantified using Qubit HS and qPCR mix pooled together for sequencing. Sequencing was performed using PE300 kit for sequencing of 18S sequencing on Illumina Miseq platform. QIIME 2 was used to remove adapter sequences, primers, and other unwanted sequence from sequence data and using Cutadapt (Bolyen et al., 2019). Finally, Pre-fitted sklearn-based taxonomy classifier, "classify-sklearn" was used to assigning taxonomy to ASVs against Silva 138 SSU database (Quast et al., 2013).

6.4 RESULTS

The experimental setups for inducing black clam spawning demonstrated a pronounced responsiveness when the factors of fluctuating salinity, temperature, and serotonin induction were combined. The significance of factors (A-temperature range, B-salinity range, and C-concentration of serotonin) and their interactions (AB, BC, and AC) were shown in Table 5.

The results show that there exists a significant difference among different levels of the factors ($p < 0.01$) viz., temperature and salinity, but the response at varied levels of serotonin was found to be non-significant ($p = 0.0751$). The significant interaction between temperature and salinity (AB) with respect to spawning (presence of fertilized

eggs) was observed ($p < 0.0001$). Conversely, the interactions between serotonin concentration and salinity/temperature (BC/AC) with response were found to be non-significant ($p = 0.8455$ & $p = 0.7422$, respectively).

Factors	Sum of Squares	Degree of Freedom	Mean Square	F-value	p-value
Model	96.99	23	4.21	122.66	<0.0001*
A-Temperature (°C)	4.41	2	2.20	64.19	<0.0001*
B-Salinity (ppt)	83.06	3	27.68	805.40	<0.0001*
C-Serotonin (mM)	0.24	2	0.12	3.50	0.0751^{ns}
AB	6.64	6	1.10	32.19	<0.0001*
AC	0.04	4	0.01	0.33	0.8455^{ns}
BC	0.11	6	0.019	0.57	0.7422^{ns}
Residual	0.30	9	0.03		
Lack of Fit	0.10	5	0.02	0.393	0.8327^{ns}
Pure Error	0.20	4	0.05		
Corrected Total	97.30	32			

Table 5. Results of ANOVA along with lack of fit for the logarithmic transformed response during the spawning of *Villorita cyprinoides* in hatchery.

The results show that there exists a significant difference among different levels of the factors ($p < 0.01$) viz., temperature and salinity, but the response at varied levels of serotonin was found to be non-significant ($p = 0.0751$). The significant interaction between temperature and salinity (AB) with respect to spawning (presence of fertilized eggs) was observed ($p < 0.0001$). Conversely, the interactions between serotonin concentration and salinity/temperature (BC/AC) with response were found to be non-significant ($p = 0.8455$ & $p = 0.7422$, respectively).

An intramuscular injection of serotonin was carried out, resulting in the successful release of spermatozoa after 20 minutes, followed by oocytes. Fertilization and early development are marked by cleavage with polar lobe formation. Trochophore larvae were observed 12 hours after spawning followed by veliger larvae after 24 hours. In addition, successful rearing of larvae up to the umboned veliger stage has been achieved (Figure 13).

However, it is noteworthy that the food intake in D-shaped larvae is comparatively less dense when compared to other larval stages in bivalves (Wu et al., 2017; Chen et al., 2020). The successful larval growth was observed until the pediveliger stage.

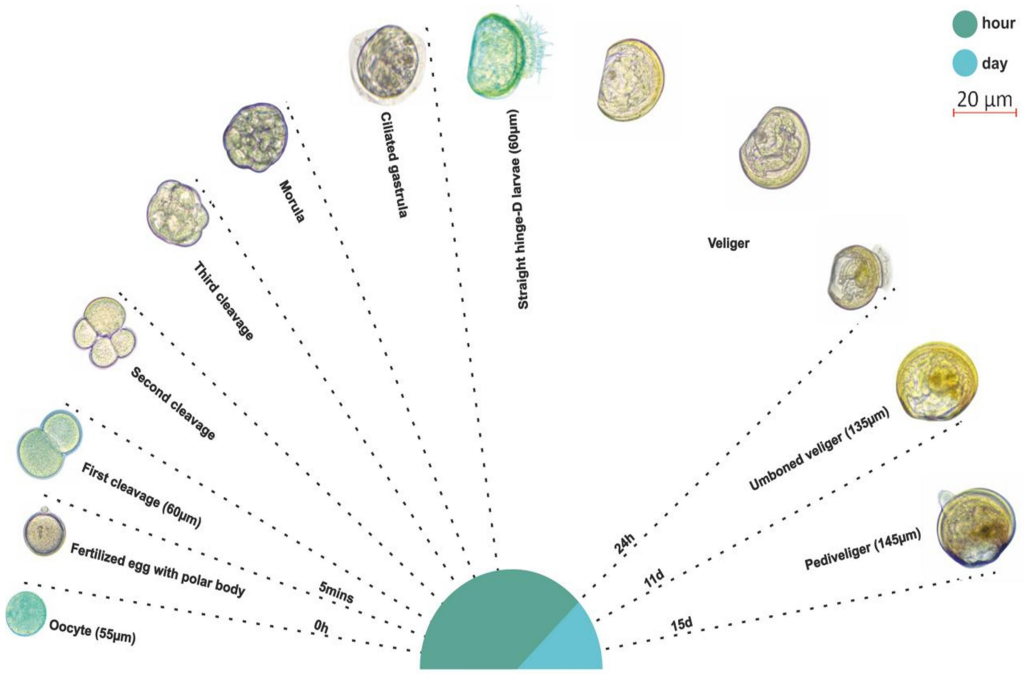

Figure 13. Developmental stages of *Villorita cyprinoides*, encompassing egg, embryonic, and early larval stages, with scale bars set at 20 µm

The growth of the bivalve was influenced by the availability and quality of food (Gosling, 2003). However, despite its potential for aquaculture, there has been limited exploration into the feeding regime of the black clam. A total of 32 phyla were identified, of which Diatomea and Dinoflagellata predominated with 22.9% and 18.04%, respectively. Dinophyceae was the dominant class within the phylum Dinoflagellata, while Mediophyceae prevailed as a class under the phylum Diatomea.

The Krona chart representing the relative abundance of taxa at various taxonomic levels and OTU richness for all phyla recovered through eDNA metabarcoding were represented in Figure 14.

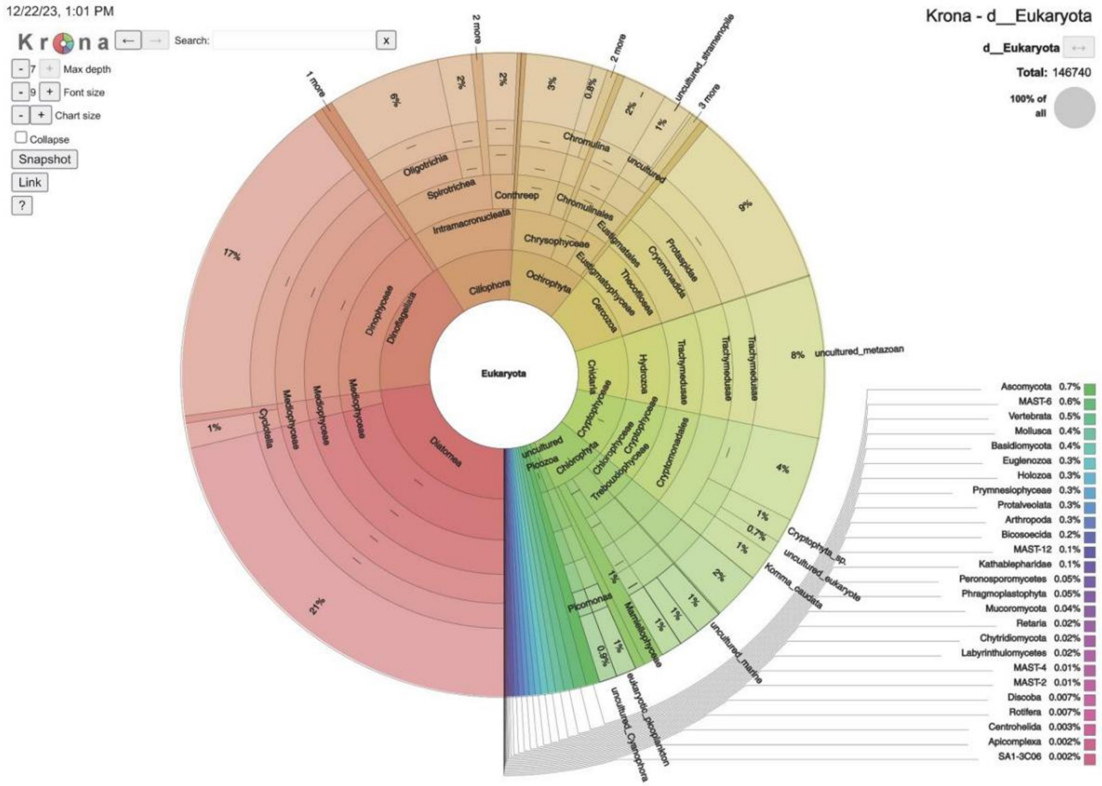

Figure 14 Krona charts represent the relative abundance of taxa at various taxonomic levels (Operational Taxonomic Units - OTUs) obtained by eDNA metabarcoding in water samples from the broodstock collection site of *Villorita cyprinoides* in the Vembanad estuary, Kerala, India.

6.5 DISCUSSION

A best knowledge on developmental stages of bivalve larvae and their feeding ground is the primary objective towards aquaculture. Induced spawning studies in bivalves are one of the easiest and practical ways to understand developmental stages. Marine invertebrates, including bivalves, exhibit distinct reproductive strategies, which can be categorized into three groups: those that breed continuously throughout the year, winter, and summer breeders (Giese & Pearse, 1974). Among these, *Villorita* falls under year-round breeder.

The present experimental evidence suggests that spawning of mature clam is determined by salinity and temperature. Although serotonin can induce spawning at any concentration, subsequent embryonic development is regulated by two other factors. The successful larval growth was observed till pediveliger stage. The timing and duration of reproductive activity and subsequent larval development are determined

by the interaction of the exogenous and endogenous factors (Fingerman, 2000). Additionally, our efforts to document the development stages of the larvae have only extended to the pediveliger stage, leaving gaps in our understanding of their natural diet. The present results of plankton dynamics can be used as a baseline data to understand the species-related nutrient profiles while the formation of microencapsulated diets (Pereira et al., 2023).

6.6 CONCLUSION

The present study paves the way for advancements in both scientific knowledge and practical applications in aquaculture. Considering the dependence of fisherman families on this fishery resource for their daily needs and harmful dredging-induced stress during peak breeding season shows the importance of sustainable management of black clam. Although further efforts are required to establish a comprehensive larval-rearing protocol, this study illuminates the scope of introducing new species into the mariculture industry and fortifying the bivalve culture essential for future growth. The result of characterization of the morphology and timing of the embryonic development process provide reference for commercial breeding of the clam whereas, identification of plankton dynamics help in aid of future artificial diet formulation for brackish water bivalve larval rearing.

Chapter 7

Conclusion and future directions

The whole research aimed to draw attention to *Villorita cyprinoides*, commonly known as the Indian black clam, which is an irreplaceable species in Indian bivalve fishery. The species with paramount importance and yet received less attention on its sustainable fishery practices. The core focus of my research centered on genetic studies of this species. This interdisciplinary research focused on population genetics, phylogenetics, trnacsriptomics, and aquaculture genetics that deals with standardization of breeding and allied genetic research. A step towards standardization of breeding and larval rearing protocols offers a significant potential for the expansion of *V. cyprinoides* aquaculture. Additionally, the development of formulated diets based on the species nutritional preferences can be done in future considering the composition of microalgal biota that can further enhance growth and survival rates of larvae and spat. The insights

gained from this study will also inform ecotoxicological monitoring *and* conservation management of estuarine ecosystems, positioning *V. cyprinoides* as a model species for future research on estuarine bivalves.

All the results from the work contribute to genetic insights essential for formulating conservation management strategies, thereby providing a leap towards sustainable fishery in the near future, benefiting both the local economy and ecosystem health.

PUBLISHED/ ACCEPTED/ COMMUNICATED ARTICLES: 4

Rahuman, S., Jeena, N.S., Asokan, P.K., Vidya, R., & Vijayagopal, P. (2020). Mitogenomic architecture of the multivalent endemic black clam (*Villorita cyprinoides*) and its phylogenetic implications. *Scientific Reports*, *10*(1), 15438. (published).

Rahuman, S., Jeena, N.S., Sebastian, W., & Sajeela K.A. (2024). Mitogenomic profiling of *Marcia recens* (Holten, 1802): A step towards taxonomic resolution of India's Ashtamudi short-neck clam. *Molluscan Researc*h, 1-10. (published).

Rahuman, S., Jeena, N.S., Sebastian, W., Varghese, E., & Asokan, P.K. (2024). Tidings from the Tides–*De novo* transcriptome assembly of the endemic estuarine bivalve *Villorita cyprinoides*. *Scientific Data*, *11*(1), 723. (published).

Rahuman, S., Jeena, N.S., Anil, M.K., Gomathi, P., Ebeneezar, S., Varghese, E., Vijayagopal, P., & Asokan, P.K. (2024). Induced spawning, larval rearing, and innate diet analysis of Indian black clam, *Villorita cyprinoides*: An alternative to resource restoration through aquaculture. *Aquaculture*, *590*, 741101. (published).

Rahuman, S., Jeena, N.S., Varghese, E., & Sajeela K.A. Genetic structure and shell geometric morphometrics of Indian black clam across its distribution range (to be submitted)

Rahuman, S., Jeena, N.S., & Sebastian, W. Phylotranscriptomics of Cyrenoidea (to be submitted)

POSTERS/PAPERS PRESENTED: 4

Participated and presented a paper, “Perplexing Taxonomic Status of the Mangrove Clam *Geloina* sp. (Family: Cyrenidae)”in World Brackish water Aquaculture Conference, BRAQCON 2019 organized and held at Central Institute of Brackish water aquaculture (CIBA), Tamilnadu, India.

Participated and presented “First Report on Induced Spawning of the Estuarine Black Clam *Villorita cyprinoides* (Gray,1825)” In 12th Indian Fisheries & aquaculture forum, 2022 organized by Tamil Nadu Dr. J Jayalalithaa Fisheries University (TNJFU) & Asian Fisheries Society Indian Branch (AFSIB), Tamilnadu, India.

Participated and presented “From *Paphia malabarica to Marcia recens*: A Taxonomic Journey of the Short-Neck Clam”, in Animal Taxonomy Summit-2023, organized by Zoological Survey of India.

Participated and presented "*De Novo* Transcriptome Assembly: Unveiling The Transcriptome Of The Endemic Estuarine Black Clam", in 'BioZion-The Biotech Capstone-Science-Society Interface'2023, organized by College of Agriculture, Vellayani, Kerala Agricultural University and SCTIMST, partnering DAAD: German Academic Exchange Service and KSCSTE.

ACHIEVEMENTS

Awarded best oral presentation under the theme 'Taxonomy' in the Animal Taxonomy Summit-2023 (ATS 2023) organized by the Zoological Survey of India held on 1st-3rd July 2023.

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